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(54) SYSTEME DE TEST IN VITRO POUR LA GAMMA-SECRETASE

(54) GAMMA-SECRETASE IN VITRO TEST SYSTEM

(57)

The invention relates to a method for identifying specific gamma-secretase inhibitors which can be used for treating neuro-degenerative diseases, in particular to a method which can be carried out in vitro. The invention also relates to a test kit which can be used for said method, in addition to the use of this test kit or method to identify substances which specifically inhibit gamma-secretase. A further embodiment relates to the use of these substances to produce a medicament for treating neuro-degenerative diseases and to pharmaceutical formulations which contain said substances.



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(54) **Titre : SYSTEME DE TEST IN VITRO POUR LA γ -SECRETASE**
(54) **Title: IN VITRO TEST SYSTEM FOR γ -SECRETASE FROM ENRICHED MEMBRANES**

(57) **Abrégé/Abstract:**

The invention relates to a method for identifying specific γ -secretase inhibitors which can be used for treating neuro-degenerative diseases, in particular to a method which can be carried out in vitro. The invention also relates to a test kit which can be used for said method, in addition to the use of this test kit or method to identify substances which specifically inhibit γ -secretase. A further embodiment relates to the use of these substances to produce a medicament for treating neuro-degenerative diseases and to pharmaceutical formulations which contain said substances.

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Abstract

The present invention falls within the scope of processes for finding specific γ -secretase inhibitors which can be used to treat neurodegenerative disorders, particularly processes which can be carried out *in vitro*. The invention also relates to a test kit with which the process according to the invention can be carried out, and the use of this test kit or of the method for finding substances which specifically inhibit γ -secretase. Another embodiment relates to the use of these substances for preparing a medicament for treating neurodegenerative disorders and pharmaceutical formulations which contain these substances.

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 γ -Secretase *in vitro* test system**Technical field of the invention**

The present invention relates to the field of methods of discovering specific γ -secretase inhibitors which can be used to treat neurodegenerative disorders, particularly methods which can be carried out *in vitro*. This invention further relates to a test kit with which the process according to the invention can be carried out, and the use of this test kit or of the method of discovering substances which specifically inhibit γ -secretase. Another embodiment relates to the use of these substances for preparing a medicament for treating neurodegenerative disorders and pharmaceutical formulations which contain these substances.

Background to the invention

The aggregation and precipitation of proteins are implicated in the origins of various neurodegenerative disorders such as Alzheimer's, Parkinson's and St. Vitus' dance ("Huntington's Chorea"). In Alzheimer's disease the amyloid- β -peptide ($A\beta$) aggregates and leads to insoluble senile plaques which constitute one of the pathological markers of the disease (Selkoe *et al*, 1996). $A\beta$ is formed by the proteolytic cleaving of a precursor protein, amyloid precursor protein (APP). Two methods of metabolising APP have been detected, the non-amyloidogenic method and the amyloidogenic method (Selkoe, 1991, 1994).

In the non-amyloidogenic metabolism of APP, α -secretase cleaves within the $A\beta$ region of the APP and thus leads to the secretion of the soluble N-terminal region of the protein (α -APPs) and, after the γ -secretase cutting has taken place, to the release of p3. By contrast, the amyloidogenic route leads to the formation of $A\beta$, two proteases generating the N-terminus (β -secretase) and the C-terminus (γ -secretase), respectively, of $A\beta$ (Haass, 1993; Selkoe, 1994).

$A\beta$ can be detected in human plasma and cerebrospinal fluid *in vivo*. In cell culture, too, secreted $A\beta$ can be detected in the cell culture supernatant of various types of cells which express or overexpress APP or fragments thereof endogenously. Both $A\beta$ production and hence the formation of amyloid plaques are influenced by various genetic risk factors. These include mutations in the homologous proteins preseniline 1 and preseniline 2 as well as in the APP itself. Analysis of these mutations on fibroblasts of Alzheimer's patients with Familial Alzheimer's Disease (FAD) showed the influence they have on the formation of $A\beta$. This was confirmed by investigations on transfected cells and transgenic animals. All mutations increase the production of $A\beta$ and in the case of the preseniline mutations lead to a selective increase in the longer $A\beta$

variant, A β 42 (Selkoe, 1996; Price, 1998). This peptide aggregates to a greater extent than the shorter form, A β 40, and is found in the diffuse plaques and together with A β 40 in the senile plaques (Lemere *et al.*, 1996; Mann *et al.*, 1996). In addition to this influence of the mutations, there are indications that the wild-type forms of the presenilins also have a fundamental function in the physiological production of A β . In neurones of mouse embryos in which the PS1 gene (PS: preseniline) has been switched off by genetic engineering, a drastic reduction in A β 40 and A β 42 was detected. In addition, the C-terminal fragments of the APP accumulate in these cells, leading to the assumption that the presenilins activate the γ -secretase or themselves have a γ -secretase activity (De Strooper *et al.*, 1998; Sisodia *et al.*, 1998). First *in vitro* test systems combined with mutation studies on conserved aspartates of preseniline 1 lead one to assume that the presenilins could be special autocatalytically active aspartate proteases which are responsible for the γ -secretase cutting in the membrane (Wolfe *et al.*, 1999).

The discussion of the identification of γ -secretase as a crucial step in the generation of A β and hence in the onset of Alzheimer's is not concluded to this day. Quite apart from this, this protease is an interesting target for intervening pharmacologically in the process of A β formation by finding inhibitors which will selectively reduce its activity. For this reason it is important to develop *in vitro* test systems, in addition to animal models and cell assays, which make it possible to test specific active substances independently of transport processes within the cell.

Wolfe *et al.* (1999) showed an *in vitro* system for measuring the activity of γ -secretase. To prepare the system, membranes from cells which stably express PS1 are worked up. They are mixed with a plasmid coding the LC99 polypeptide and subjected to an *in vitro* reaction of transcription/translation in the presence of ^{35}S -methionine, forming the γ -secretase substrate C99. The mixture is then incubated under suitable conditions, during which time the C99 fragment of APP is proteolytically cleaved from the γ -secretase and the breakdown products are detected by gel electrophoresis after immunoprecipitation. LC99 within the scope of the invention denotes a fusion protein between the γ -secretase substrate C99 and a signal sequence (L: "leader"; Shoji *et al.*, 1992).

Brief summary of the invention

The present invention falls within the scope of methods of discovering specific γ -secretase inhibitors which can be used to treat neurodegenerative disorders, particularly methods which

can be carried out *in vitro*. This invention further relates to a test kit with which the process according to the invention can be carried out, and the use of this test kit or of the method of discovering substances which specifically inhibit γ -secretase. Another embodiment relates to the use of these substances for preparing a medicament for treating neurodegenerative disorders and pharmaceutical formulations which contain these substances.

Figures

Fig. 1: Characterisation of the microsomal fraction SIII

The H4-ind/APP-LC99 cells were left to grow in the absence of doxycycline for three days in order to induce the expression of LC99. Post Nuclear Supernatant (PNS) was prepared as described and further concentrated using sucrose step gradients (Taylor *et al.* 1997)

A. Concentration of the γ -secretase substrate C99 in the microsomal fraction SIII. Aliquots (in each case 30 μ g) of the PNS and the microsomal fraction (SIII) were placed on a 12% SDS-polyacrylamide gel. For this purpose, PNS was prepared from H4-ind/APP-LC99 cells which had grown in the presence and absence of doxycycline. The proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Poly Screen, New Life Science) and C99 was detected with the polyclonal antibody 5818, which is directed against the last 20 amino acids of the C99 (diluted 1:1000; alternative antibodies with the same activity: Product Number SAD 3138, Labgen).

B. Concentration of the presenilines in the microsomal fraction SIII.

Aliquots (in each case 30 μ g) of the PNS and fractions of the sucrose step gradient were loaded onto a 12% SDS polyacrylamide gel and the proteins separated were transferred to a PVDF membrane (Poly Screen, New Life Science). In order to detect the two PS proteins either the monoclonal antibody BL3D7 (PS1 CTF-specific; 1:3000; Steiner *et al.*, 1999) or the monoclonal antibody BI-HF5C (PS2 CTF-specific; 1:3000; Steiner *et al.*, 1999) was used. It was shown that CTFs of PS1 and PS2 had been concentrated in the SIII fraction. It was not possible to tell whether the protein band observed at a molecular weight of about 46-50 kDa in each case was full length PS1 or PS2.

C. Characterisation of the SIII fraction by the detection of marker proteins which are characteristic of certain compartments

Aliquots (in each case 30µg) of the PNS and fractions of the sucrose step gradient (9) were loaded onto a 12% SDS polyacrylamide gel and the proteins separated were transferred to a PVDF membrane (Poly Screen, New Life Science).

To demonstrate the concentration of membranes of the endoplasmatic reticulum in the SIII fraction an anti-calreticulin antibody (Stressgen Biotechnologies; 1:2000) was used. The distribution of endosomal membranes in the gradient was shown with the aid of anti-rab5 antibodies (Transduction Laboratories Inc.).

Fig. 2: Cell-free generation of Aβ 40 and Aβ 42 from isolated microsomes

A. *De novo* Aβ generation in a cell-free γ-secretase test system with isolated microsomes of H4 cells, stably transfected with APP-LC99. Microsomes were prepared as described (Taylor *et al.*, 1997) (SIII-fraction) and incubated at 37°C or 4°C for 4 hours under neutral conditions (pH 6.8). After incubation of the substrate C99 the products Aβ40 and Aβ42 were immunoprecipitated with the antibodies 6E10 and 4G8 (Senetek, Great Britain; Galli *et al.*, 1998). In this way the substrate/product ratio could be estimated. To precipitate the Aβ40 and Aβ42 peptides alone the specific antibodies BI.40 and BI.42 were used. The precipitated proteins were separated with a Tris-Bicine gel, transferred to a nitrocellulose membrane and made visible with the antibodies 6E10 and 4G8, using a highly sensitive Western Blot procedure (Ida *et al.*, 1996). To determine the basal intracellular Aβ content, immunoprecipitations were carried out directly with the microsomal fraction stored at - 80°C (Lane c). The Aβ40 and Aβ42 generated *in vitro* migrates with the synthetic Aβ peptides. Lower part: longer exposure

B. Time-dependency of the generation of Aβ *in vitro*.

The SIII fraction was prepared as described and incubated for the specified time at 37°C or 4°C under neutral conditions (pH 6.8). Aβ peptides were immunoprecipitated with the specific antibodies BI.40 and BI.42. The precipitated proteins were separated by Tris-Bicine gel electrophoresis (Klafki *et al.*, 1996) and then detected using a highly sensitive Western blot procedure with the antibodies 6E10 and 4G8 (Ida *et al.*, 1996). The synthetic Aβ40 and Aβ42 peptides were used as the standard. After 3-4 hours' incubation at 37°C the *de novo* generation of Aβ reached a peak.

Fig. 3: The γ-secretase cleavage product Aβ is degraded by a Ca²⁺-dependent protease

The SIII fraction was produced as described and incubated under standard conditions (37°C; pH 6.8; 4 hours) in the presence or absence of cation chelators such as EDTA or BAPTA. As a control the microsomes were incubated at 4 °C in the presence of EDTA. Aβ peptides were immunoprecipitated with the specific antibodies BI.40 and BI.42 and detected by Western blot with the antibodies 6E10 and 4G8 (Senetek, Great Britain; Galli *et al.*, 1998) as described. The synthetic peptides Aβ40 and Aβ42 were used as standard. All the reactions were carried out three times. In the absence of a chelator the *de novo* Aβ production is drastically reduced. Both EDTA and BAPTA, which chelates only Ca²⁺ ions, result in a larger quantity of Aβ *de novo*. This can be explained by the fact that Aβ is degraded again by a Ca²⁺-dependent protease immediately after production.

Fig. 4: γ-Secretase is a transmembrane protease.

The SIII fraction was prepared as described and incubated under standard conditions (pH 6.8; 5 mM EDTA; 4 hours) at 37°C or 4°C as a control. The microsomal membranes were either washed with a highly saline solution (1 M KCl) or extracted with Na₂CO₃ in order to remove weakly bound proteins from the microsomal membranes. To do this the pelleted membranes were resuspended in KCl buffer (1 M KCl, 250 mM sucrose, 20 mM Hepes, pH 6.8) and incubated for 30 min at 4°C. For the Na₂CO₃ extraction the membranes were homogenised in 100 mM Na₂CO₃, pH 11.5, and incubated for 30 min at 0°C. The membranes were incubated at 220,000 g for 1 hour at 4°C, carefully washed with cold water and washed with 1 ml of reaction buffer (9) during the homogenisation. The membranes were centrifuged as described above and resuspended in reaction buffer. Aliquots were frozen in liquid nitrogen. Aβ peptides were immunoprecipitated with the specific antibodies BI.40 and BI.42 and detected by Western blot with the antibodies 6E10 and 4G8 as described. The synthetic peptides Aβ40 and Aβ42 were used as the standard. All the reactions were duplicated. Independently of the pretreatment of the membranes the content of Aβ generated *de novo* was virtually identical. These data lead one to assume that γ-secretase is a protease which is either fixedly bound to the membrane or is an integral membrane protein.

Fig. 5: The optimum pH for the γ-secretase activity is between pH 6.8 and 7.4

The SIII fraction was prepared as described and incubated under standard conditions (pH 6.8; 5 mM EDTA; 4 hours) at the pH values specified. Aβ-peptides were immunoprecipitated with the

specific antibodies BI.40 and BI.42 and detected by Western blot with the antibodies 6E10 and 4G8 as described. All the reactions were duplicated. As a control the *in vitro* reaction was carried out at 4°C and pH 6.8. The optimum pH for the *in vitro* γ -secretase activity is in the neutral range between pH 6.8 and pH 7.4. A sharply reduced γ -secretase activity was found both under slightly acidic and under basic pH conditions.

Fig. 6: Effect of a possible γ -secretase inhibitor in the cell-free γ -secretase test system.

The SIII fraction was prepared as described and incubated at a pH of 6.8 under standard conditions in the presence or absence of different concentrations of compound A (Fig.: A) (pH 6.8; 5 mM EDTA; 4 hours). A β peptides were immunoprecipitated with the specific antibodies BI.40 and BI.42 and detected by Western blot with the antibodies 6E10 and 4G8 (Senetek, Great Britain; Galli *et al.*, 1998) as described. All the reactions were performed in duplicate or triplicate. As a control the reactions were carried out at 4°C.

A. Compound A exhibited an inhibition of the *in vitro* generation of A β dependent on concentration. The A β generated *de novo* was quantified with the Chemiluminescence Imaging System (Biorad) and this is shown in the lower part.

B. Compound A exhibited a concentration-dependent reduction in extracellular A β 40 and A β 42

Compound A was also active in a cellular test system in which the extracellular content of A β 40 and 42 secreted by the U373 cell line (U373: ATCC No. HTB 14) is determined. This cell line U373/APP751 is an Astrocytoma cell line which overexpresses human APP₇₅₁ and secretes large amounts of A β 40 (~1000pg/ml/ 4 hours with 5×10^7 cells in 15 ml of medium) and A β 42 (~100pg/ml/ 4 hours with 5×10^7 cells in 15 ml of medium). The assay is done using ELISA (ELISA: "Enzyme linked immunosorbent assay" (Steiner *et al.*, 1998). The A β 40 secretion was sharply reduced by compound A in a concentration-dependent manner, slightly increasing the secretion of A β 42 at subinhibitory doses and then also inhibiting it at higher doses.

Fig. 7: Effect of a γ -secretase inhibitor as described in the cell-free γ -secretase test system.

The SIII fraction was prepared as described and incubated at a pH of 6.8 under standard conditions in the presence or absence of various concentrations of the compound MG132 (Biomol Order No. : PI-102; De Strooper *et al.* 1999) (pH 6.8; 5 mM EDTA; 4 hours). A β

peptides were immunoprecipitated with the specific antibodies BI.40 and BI.42 and detected by Western blot with the antibodies 6E10 and 4G8 (Senetek, Great Britain; Galli *et al.*, 1998) as described. All the reactions were duplicated. As a control the reactions were carried out at 4°C or at 37°C without any inhibitor.

- A) The compound MG132 displayed a concentration-dependent inhibition of the *in vitro* generation of A β .
- B) Quantifying the A β generated *de novo* was done using the Chemiluminescence Imaging System (Biorad) and is shown in the lower part.

Fig. 8 : Characterisation of the microsomal fractionation of H4indLC99 cells

The H4-ind/APP-LC99 cells were allowed to grow for three days in the absence of doxycycline in order to induce the expression of LC99. The fractions were prepared as described (Schroter *et al.* 1999).

A) Detection of marker proteins which are characteristic of certain compartments

Aliquots (in each case 30 μ g) of the fractions were loaded onto a 12% SDS-polyacrylamide gel and the separated proteins transferred onto a PVDF membrane (Poly Screen, New Life Science). To show the concentration of membranes of the endoplasmatic reticulum in the Mi fraction an Anti-PDI antibody (Stressgen Biotechnologies; 1:2000) was used. As a comparison the SIII fraction of the first microsomal fractionation was also added. PDI is concentrated in the microsomal fraction. The distribution of lysosomal membranes in the fractions was shown using Anti-CathepsinD antibodies (Transduction Laboratories Inc.; 1:1000). The microsomal fraction is free from lysosomal proteins. As a comparison, the PNS of the H4indLC99 cells was also added.

B) Cell-free formation of A β in the microsomal fraction

Microsomes (Mi-fraction) were prepared as described and incubated at a pH of 6.8 under standard conditions (pH 6.8; 5 mM EDTA; 4 hours).

A β peptides were with the specific antibodies BI.40 and BI.42 immunoprecipitated and detected by Western blot with the antibodies 6E10 and 4G8 (Senetek, Great Britain; Galli *et al.*, 1998) as described. The incubations at 37°C were carried out in duplicate. The formation of A β takes place in the microsomal fraction, as well as in the endosomal fraction.

Detailed description of the invention

The problem of providing an improved test system is solved by the present invention within the scope of the specification and claims. According to the invention, an *in vitro* test system for discovering substances which are capable of specifically inhibiting γ -secretase is provided. In another embodiment of the invention a test kit for discovering substances which are capable of specifically inhibiting γ -secretase is disclosed. Another embodiment of the invention relates to the use of the process according to the invention or the test kit according to the invention for discovering substances which are capable of specifically inhibiting γ -secretase. Moreover, substances are prepared which can be found using the process according to the invention or the test kit according to the invention. Another embodiment relates to the use of these substances for preparing a medicament for treating neurodegenerative disorders and pharmaceutical formulations which contain these substances.

The process according to the invention uses purified membranes which are isolated from cells which detectably express a substrate of γ -secretase and have a γ -secretase activity. By γ -secretase is meant, within the scope of this invention, a protein which has the property of proteolytically cleaving APP or fragments thereof, particularly the C99 peptide (Shoji *et al.*, 1992), in p3 or A β . Thus, any cells in which the skilled man can detect a substrate of γ -secretase or the cleavage products thereof by Western Blot and the use of specific antibodies are suitable for the process according to the invention. Suitable membranes are any membranes in which the skilled man can detect a substrate of γ -secretase by Western Blot and the use of specific antibodies, preferably lysosomal and endosomal membranes, most preferably microsomal membranes. Methods of specifically purifying membranes are known to the skilled man from Methods in Enzymology, Vol. 219, title: Reconstitution of intracellular transport, and the book "Biochemische Arbeitsmethoden" [Biochemical working methods], T.G. Cooper, published by De Gruyter, 1981. In order to carry out the process according to the invention, cells can be transfected with a DNA sequence (DNA = deoxyribonucleic acid) which codes for a substrate of γ -secretase, as described in a non-limiting embodiment in the example. In one embodiment by way of example, the expression of this substrate can be induced by the removal of doxycycline. The cells can be opened and a post nuclear supernatant (PNS) can be prepared, which is worked up further in order to isolate the microsomal fraction, for example. This fraction can be incubated under suitable conditions and the formation of the product of the reaction of γ -secretase with a suitable substrate can be determined by immunoprecipitation and subsequent detection using suitable

antibodies by the Western Blot method. The γ -secretase substrate was found in the PNS and in a higher concentration in the microsomal fraction (Fig. 1A). The C-terminal fragments (CTF) of PS1 and PS2 were also concentrated in the SIII fraction (Fig. 1B). The microsomal fraction contained no endosomal membranes, but ER and Golgi compartments were concentrated (Fig. 1C). A β generated *de novo* was found in the microsomal fractions which had been incubated at 37 °C (Fig. 2A). Incubation at 4 °C prevented the *de novo* formation of A β . Small amounts of A β were the result of the existence of intracellular A β in the freshly prepared microsomal fractions (Fig. 2A, trace c). The *de novo* formation of A β was dependent on time and reached its peak after 3 to 4 hours' incubation (Fig. 2B). However, an evaluation of the substrate/ product equation showed that the *in vitro* generation of A β was not an efficient proteolytic reaction (Fig. 2A). The incubation of the microsomal membranes in the absence of EDTA led to a dramatic reduction in the *in vitro* generation of A β (Fig. 3). The calcium chelator BAPTA had the same effect. This leads to the assumption that a Ca²⁺-dependent protease present in the same fraction breaks down A β . The treatment of the microsomal membranes with 1 M KCl in the buffer or extraction of these membranes with 0.1 M Na₂CO₃ pH 11.5 before the γ -secretase test system is carried out shows that the γ -secretase has to be located on the membrane or at least firmly bound to the membrane (Fig. 4). In contrast to data published previously (Wolfe *et al.*, 1999) the optimum pH determined for the γ -secretase activity using this test system is between 6.8 and 7.4 (Fig. 5). There was no *in vitro* generation of A β either at a more basic pH (pH 8.0 to 8.5) or at a more acid pH (pH 6.0 to 6.4).

In one embodiment according to the invention, the purified membranes described, particularly microsomal membranes, are mixed with a suitable reaction buffer and a test substance. By test substance is meant, for the purposes of the invention, any substance which is to be tested to find out whether it might have an inhibitory effect on the γ -secretase activity. Then the mixture is incubated under conditions in which the substrate of the γ -secretase is cleaved in the absence of the test substance. The quantity of the cleavage product formed is then determined and the value obtained is compared with the value obtained in the absence of the test substance. If the amount of cleavage product formed in the presence of the test substance is less than in the absence of the test substance, the test substance inhibits the formation of the cleavage product and the test substance is an inhibitor of γ -secretase. In the prior art, compounds are identified, *inter alia*, which are referred to as specific γ -secretase inhibitors and had an inhibitory effect on the

secretion of A β 40 and A β 42 without influencing the formation of A β . The γ -secretase-test system according to the invention can now advantageously be used to identify and validate specific γ -secretase inhibitors and distinguish them from inhibitors that prevent the secretion of A β 40 and A β 42. A compound was tested using the test system according to the invention as described in the Example. The compound A ([α S-(α R*, γ R*, δ R*)] N-butyl- γ -hydroxy- α -(1-methylethyl)- δ -[(4-methylpentyl)amino]cyclohexane hexanamide-hydrochloride; cf. Example 20 of EP 778 266 A1) was prepared as described in Example 20 of European Patent Application EP 778 266 A1 and exhibited a concentration-dependent inhibition of A β generation with an IC50 value of about 6 μ M (Fig. 6A). Similar results were obtained in a test system which measures secreted A β 40 and A β 42 in a quantitative ELISA, which is specific to both A β peptides (Steiner *et al.*, 1998). Here, an Astrocytoma cell line (U373) is used which overexpresses wild-type APP751 and secretes detectable amounts of both species of A β . A concentration-dependent reduction in the amount of extracellular A β 40 and A β 42 was observed after overnight treatment with compound A (Fig. 6B).

In one embodiment of the invention, cells are cultivated which express an endogenous polypeptide which is a substrate of γ -secretase. The term endogenous within the scope of this invention means that this cell or cell line expresses the polypeptide referred to in a sufficient quantity without any need for further manipulation, e.g. by genetic engineering methods. Within the scope of the invention a sufficient quantity of substrate of γ -secretase denotes a quantity which will produce a detectable signal standing out from the background in an established biochemical method of detection (e.g. ELISA, Western Blot) using specific antibodies.

In a particularly preferred embodiment of the invention cells are cultivated which express an exogenous polypeptide which is a substrate of γ -secretase. The term exogenous within the scope of this invention means that this cell or cell line is manipulated by genetic engineering methods so that it expresses the γ -secretase substrate. If the cell or cell line contains the γ -secretase substrate even without these manipulations, this term means that the quantity of γ -secretase substrate is measurably increased compared with the value without any manipulation. In order to prepare a cell which exogenously expresses the γ -secretase substrate a nucleotide sequence which codes for the amino acid sequence of the γ -secretase substrate can be inserted in a suitable expression cassette of a eukaryotic expression vector. Suitable expression cassettes have a

promoter which is functional in eukaryotic hosts such as, for example, the cytomegalovirus promoter (CMV promoter) and a functional polyadenylation signal, e.g. from the SV40 virus (Simian Virus; abbr. SV). Suitable expression vectors are vectors which can replicate in eukaryotic hosts, i.e. have a functional origin of replication. These expression vectors may be present episomally after the transfection or may be integrated into the genome if they carry suitable sequences that make integration possible.

In a preferred embodiment, an expression system is used that makes it possible to induce the expression of the exogenous polypeptide; various systems may be used here such as e.g. the "Tet-on"- or "Tet-off" system (US-Patent 5,464,758; Gossen and Bujard, 1992, 1995, marketed by Clontech, Heidelberg) or the LacSwitch system (see U.S. Patent, 5,589,392; sold by Stratagene). In a particularly preferred embodiment of the invention the expression of the γ -secretase substrate is induced by the removal of tetracycline or doxycycline ("Tet-Off" system). To do this the nucleotide sequence which codes the amino acid sequence of the γ -secretase substrate is cloned behind at least one binding site of the tetracycline repressor. In addition, another nucleotide sequence can be found on the same plasmid, another plasmid or chromosomally integrated, coding for a fusion protein between the Tet repressor and an acidic, activating domain which is constitutively expressible. By an acidic, activating domain is meant a protein domain which has a high proportion of acidic amino acids and has the capacity to mediate the transcription of a gene when the domain is placed in a suitable position in the transcription complex located in front of the gene. This capacity can be determined by the known "one-hybrid assay". In this method, a DNA-binding domain is fused with a domain which is to be investigated, the DNA-binding domain binding to a sequence located in front of a reporter protein, and the activity of said reporter protein being measured (Clontech, Heidelberg). In the case described above the expression of the γ -secretase substrate will not take place if the concentration of tetracycline or a tetracycline derivative such as e.g. doxycycline in the cell exceeds a certain level, as the tetracycline repressor binds tetracycline or the derivative thereof, and consequently does not bind to its binding site in the DNA and therefore does not induce the transcription of the gene inserted behind this binding site which codes for the γ -secretase substrate. In the absence of tetracycline or a derivative thereof the fusion protein between Tet repressor and the acidic activating domain binds to its DNA binding site and induces the

transcription of the gene inserted behind it which codes for the γ -secretase substrate. This ensures that controlled and targeted expression of the γ -secretase substrate takes place.

In one embodiment of the invention the γ -secretase substrate is the amyloid precursor protein (APP) or a fragment thereof provided that it contains the γ -secretase cutting site. In a preferred embodiment of the invention the γ -secretase substrate is the C99 fragment of the amyloid precursor protein (Shoji *et al.*, 1992).

The γ -secretase substrate may be generally membrane-associated, but is preferably membrane-based. By membrane-based is meant, within the scope of this invention, that the substrate is an integral part of the membrane. By membrane-associated is meant within the scope of this invention that the substrate is bound to the surface of the membrane or to integral membrane proteins. This definition of membrane-associated substrates is also intended to cover substrates which interact with the hydrophobic part of the membrane via chemical groupings added on by post-translational modifications. Moreover, the term membrane-associated substrate is also intended to include substrates which interact with the hydrophobic part of the membrane via amino acid side chains, albeit to a lesser extent than the integral membrane proteins.

Prostaglandin synthetase may be mentioned here by way of example.

In another embodiment of the invention the γ -secretase substrate is a fusion protein of a reporter protein with the amyloid precursor protein or a fragment thereof provided that it contains the γ -secretase cutting site. In a preferred embodiment the γ -secretase substrate is a fusion protein between a reporter protein and the C99 fragment. For the purposes of this invention a reporter protein is a protein which has the ability to generate an easily detectable signal and correlates the quantity thereof with the quantity of the cleavage product in question. The signal is generated either by determining the enzymatic activity of the reporter protein with easily detected substrates or by measuring the intensity of the fluorescence of the reporter protein. Examples of reporter proteins are green fluorescent protein (GFP; cf. e.g. WO95/07463) or derivatives thereof that fluoresce at different wavelengths or enzymes such as luciferase, secretory alkaline phosphatase or β -galactosidase. In this embodiment of the invention the fusion protein of the cleavage product with the reporter protein is separated from the fusion protein of the uncleaved γ -secretase substrate with the reporter protein by immunoprecipitation, for example. This may be carried out with antibodies that selectively recognise the cleavage product. Then the quantity of reporter protein is determined by the processes mentioned above which are independent of its

qualities. The fusion proteins referred to may be prepared by current genetic engineering methods (Sambrook *et al.*, 1989) if the DNA coding for the reporter protein and the γ -secretase substrate is available. The DNA which codes for the reporter proteins may be obtained, for example, from commercial suppliers such as Clontech of Heidelberg, and inserted into the
5 desired vectors by standard methods (Sambrook *et al.*, 1989). The DNA which codes for the γ -secretase substrate or for the C99 fragment can be obtained from suitable gene banks using standard methods (Sambrook *et al.*, 1989).

The invention may be performed using any cells or cell lines known to the skilled person, particularly eukaryotic cells or cell lines. Cells or cell lines used in neurological or
10 neurobiological research are preferred, e.g. mammalian cells or cell lines such as H4, U373, NT2, HEK 293, PC12, COS, CHO, fibroblasts, myeloma cells, neuroblastoma cells, hybridoma cells, oocytes, embryonic stem cells. Also suitable are insect cell lines (e.g. using Baculovirus vectors such as pPbac or pMbac (Stratagene, La Jolla, CA)), yeast (e.g. using yeast expression vectors such as pYESHIS (Invitrogen, CA)), and fungi.

15 Cells or cell lines of neuronal or glial origin are particularly preferred. In one particularly preferred embodiment of the invention the cells used are H4 cells, human neuroglioma cells from the brain which are deposited under ATCC Number HTB-148 at the "American Type Culture Collection (ATCC)", in Manassas, Virginia, USA.

In a preferred embodiment of the invention purified cell membranes are used, preferably
20 intracellular membranes, most preferably purified lysosomal or endosomal membranes. Particularly preferably, microsomal membranes are used which are purified from the cells used by lysing the cells, removing the cell nuclei, purifying using sucrose density gradients, re-sedimenting by ultracentrifugation, homogenising, re-sedimenting by ultracentrifugation and re-homogenising. Standard methods of purifying membranes are described in Methods in
25 Enzymology, Vol. 219, and in the book "Biochemische Arbeitsmethoden" [Biochemical Working Methods], T.G. Cooper, published by De Gruyter, 1981. Theoretically, ultracentrifugation with gradients of sucrose, metrizamide, ficoll and iodoxanol is suitable for purifying the membrane.

In a preferred embodiment of the present invention the reaction buffer has a pH value in the
30 range from 5-10, preferably in the range from 6.0 to 8.0, particularly preferably from 6.8 to 7.4 and also contains at least one membrane stabiliser. By membrane stabiliser is meant within the scope of this invention a substance which prevents the aggregation of the membranes. By

membrane aggregation is meant within the scope of this invention the clumping together or aggregation and possible subsequent fusion of vesicles or liposomes or multilamellar structures. This may be determined by the experimentally measurable increase in turbidity or light diffraction of a solution or suspension being investigated, while the properties of a substance in terms of its membrane-stabilising activity may also be determined in this way. The membrane stabiliser within the scope of this invention is preferably sucrose or sorbitol, although the skilled person may replace these with substances of equivalent activity. Preferably, the concentration of the membrane stabiliser in the reaction buffer is between 200 and 1000 mM, preferably between 200 and 500 mM, most preferably between 200 and 300 mM.

10 In a particularly preferred embodiment of the process according to the invention the reaction buffer additionally contains a complexing agent, preferably for divalent ions. This may be, for example, ethylene-diamine-tetraacetic acid (EDTA) or a salt thereof, 1,2-bis(o-aminophenoxy)ethane-N, N, N', N'-tetraacetic acid (BAPTA) or a salt thereof or ethyleneglycol-bis(b-aminoethyl)-N,N,N',N'-tetraacetic acid (EGTA) or a salt thereof. The
15 complexing agent is preferably in a concentration of 0.1 to 20 mM, preferably 5 to 10 mM. The term complexing agent denotes compounds which as ligands are capable of forming complexes, i.e. particularly complexing and masking metals, particularly divalent metal ions. The term is often used as a synonym for chelating agents. In the process according to the invention other complexing agents may also be used.

20 In one embodiment of the invention an ATP-regenerating system is also added to the reaction mixture. This system contains adenosine triphosphate (ATP), guanosine triphosphate (GTP), phosphocreatine, creatine phosphokinase, preferably in a concentration of 1 mM ATP, 0.1 mM GTP, 8 mM phosphocreatine, 31 mM creatinephosphokinase at a pH value in the neutral range, preferably between 6.8 and 7.2, most preferably at a pH of 7.0.

25 In a preferred embodiment of the invention the quantity of the cleavage product is determined by immunoprecipitation or Western Blot, preferably by a combination of immunoprecipitation and Western Blot. The immunoprecipitation and the Western Blot are carried out as described by Ida *et al.* (1996). By Western Blot is meant a method in which proteins are separated by gel electrophoresis, usually polyacrylamide gel electrophoresis, according to their charge in their
30 native or usually in their denatured state, transferred onto carriers such as e.g. nitrocellulose or polyvinylidene difluoride and then detected using antibodies. In another embodiment of the

invention the quantity of the cleavage product is determined by enzyme immunoassay (enzyme-linked immunosorbent assay; abbr.: ELISA) (Steiner *et al.*, 1999) or by mass spectrometry.

In another embodiment of the invention the quantity of the cleavage product is determined by measuring the quantity of reporter protein fused to the cleavage product, after it has been

5 purified of the fusion protein between the uncleaved γ -secretase substrate and reporter protein.

The quantity of luciferase fused to the cleavage product, secretory alkaline phosphatase or β -galactosidase is determined by measuring the enzymatic activity of the reporter protein after the addition of substrate. In another embodiment of the invention the amount of green fluorescent protein or a derivative thereof is determined by measuring the intensity of the fluorescent light.

10

A preferred embodiment of the invention is a test kit according to the invention for finding substances which are capable of specifically inhibiting γ -secretase. A test kit is a collection of all the components for the process according to the invention. Some far from exhaustive examples of other elements for carrying out the process according to the invention are containers such as, for example, 96-well plates or microtitre plates, test tubes, other suitable vessels, surfaces and

15

substrates, membranes such as nitrocellulose filters, washing reagents and buffers or the like. Similarly, a test kit may contain reagents which may have bound antibodies such as e.g. labelled secondary antibodies, chromophores, enzymes (e.g. conjugated to antibodies) and the substrates thereof or other substances capable of binding antibodies. The test kit according to the invention

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contains at least purified cell membranes from cells which exhibit γ -secretase activity and contain a substrate of γ -secretase, and reaction buffer. In a preferred embodiment the membranes are purified intracellular membranes, preferably lysosomal or endosomal, most preferably microsomal membranes. Most preferably, the membranes have been purified from cells which exogenously express a substrate of γ -secretase. In a preferred embodiment of the invention the reaction buffer has a pH in the range from 5-10, preferably in the range from 6.0-8.0, most preferably in the range from 6.8 to 7.4, and contains as other components at least one membrane stabiliser according to the invention as described above. Preferably, the concentration of the membrane stabiliser, which is preferably sucrose or sorbitol, in the reaction buffer is between 200 and 1000 mM, preferably between 200 and 500 mM, most preferably between 200 and 300

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mM. Most preferably, the reaction buffer additionally contains a complexing agent according to the invention, preferably for divalent ions. This may be as described above, e.g. EDTA, BAPTA or

EGTA or a salt thereof. The complexing agent is present in a concentration of 0.1 to 20 mM, preferably 5 to 10 mM.

In one embodiment of the invention the test kit additionally contains an ATP-regenerating system according to the invention as described above, which can be added to the reaction mixture. In another embodiment of the invention the test kit contains antibodies which make it possible to determine the quantity of cleavage product by immunoprecipitation or Western Blot, preferably by a combination of immunoprecipitation and Western Blot.

In a preferred embodiment of the invention the process according to the invention or the test kit according to the invention is used to find substances which are capable of specifically inhibiting

γ -secretase. In another embodiment a substance is prepared which can be found using the process according to the invention or the test kit according to the invention and which specifically inhibits the proteolytic cleaving of a γ -secretase substrate. The substance according to the invention may be used for preparing a medicament for treating neurodegenerative disorders, particularly Alzheimer's disease. In addition, pharmaceutical formulations are prepared which contain a substance according to the invention and a pharmaceutically acceptable carrier.

A pharmaceutically acceptable carrier may contain physiologically acceptable compounds which increase the stability or absorption of the substance according to the invention, for example.

Such physiologically acceptable compounds contain e.g. carbohydrates such as glucose, sucrose or dextrane, antioxidants such as ascorbate or glutathione, chelating agents, proteins with a low molecular weight or other stabilisers (cf. e.g. Remington's Pharmaceutical Sciences (1990)).

Anyone skilled in the art knows that the choice of a pharmaceutically acceptable carrier including a physiologically acceptable compound depends for example on the route of administration.

Example 1- Using the test system:**1.1 Preparation of a suitable cell line**

H4 Neuroglioma cells (Accession number HTB 148 at the "American Type Culture Collection", Manassas, Virginia, USA) were transfected under standard conditions with the regulator plasmid pUHD15-1neo (pUHD15-1 with neomycin resistance gene), which carries the gene for a tetracycline repressible transactivator (Gossen and Bujard, 1992, 1995). By transient transfection experiments an individual clone was selected for the second stable transfection with the APP-LC99 construct, having a strictly regulated and strongly inducible transient expression of a reporter gene (pUHD10-3/ SEAP; SEAP: secretory alkaline phosphatase). To prepare the APP-LC99 construct a sequence which contains the N-terminal signal sequence and the last 99 amino acids of the APP (Shoji *et al.*, 1992), was cloned into the tetracycline-controlled expression vector pUHD10-3 via BamHI and SacII restriction cutting sites. This construct was called pUHD10-3/APP-LC99. The cell clone obtained as described above was co-transfected with 10 µg of pUHD10-3/ APP-LC99 and 1 µg of pTK-Hyg (Clontech, Heidelberg; Gene bank accession number U40398) and selection was carried out using the Fugene transfection system made by Boehringer Mannheim in accordance with the manufacturer's instructions. Individual hygromycin-resistant cell clones were investigated for the inducible expression of LC99 by the removal of doxycycline and subsequent immunofluorescence and/or Western Blot with an APP-CTF-specific antibody. The selected clone was called H4-ind/ APP-LC99.

1.2 Working up a microsome fraction from H4 LC99 cells

The H4-ind/ APP-LC99 cells were grown until confluent on 15 cm Petri dishes at 37 °C at 5% CO₂ with DMEM medium (DMEM: "Dulbecco's Modified Eagle Medium", sold by BioWittacker) and 10% foetal calf serum (FCS), 1% glutamine, 1% penicillin and streptomycin in the absence of doxycycline. By the removal of doxycycline the expression of the fusion protein was induced. All the steps of preparing the postnuclear supernatant were carried out on ice or at 4°C. After the addition of 2 ml of PBS per Petri dish the cells were removed from the Petri dishes with a cell scraper. After centrifugation at 500 g for 10 min the cells were carefully resuspended in HIS buffer (250 mM sucrose, 5 mM imidazole, 10 mM HEPES pH 6.8), centrifuged again at 1400 g for 10 min and then resuspended in 300 µl of HIS⁺ buffer (HIS buffer with 5 mM EDTA) per Petri dish. The homogenised cell material was forced through a 22

gauge needle using a 1 ml syringe and the lysing of the cells was monitored by phase contrast microscopy. The cell lysate was centrifuged at 2500 g for 10 min in order to separate the supernatant from the intact cells and the cell debris.

The PNS was worked up further with a sucrose step gradient (Taylor *et al.*, 1997), first adjusting it to 100 mM KH_2PO_4 / K_2HPO_4 , pH 6.8. All the sucrose solutions contained 100 mM KH_2PO_4 / K_2HPO_4 , pH 6.8, 5 mM MgCl_2 and the protease inhibitors leupeptin (10 $\mu\text{g}/\text{ml}$) and aprotinin (10 $\mu\text{g}/\text{ml}$). The gradient contained steps of 1.3 M, 0.86 M and 0.5 M sucrose, which were overlaid with the PNS and then centrifuged at 100,000 g for 1.5 hours at 2 °C. The intermediate layer between 1.3 M and 0.86 M sucrose is the microsomal fraction SIII. In order to pellet the membranes, the SIII fraction was adjusted to 250 mM sucrose with 100 mM KH_2PO_4 / K_2HPO_4 , pH 6.8, and centrifuged at 220,000 g for 20 min at 4°C. The membranes were washed with reaction buffer (250 mM sucrose, 50 mM KCl, 2,5 mM magnesium acetate, 20 mM HEPES, pH 6.8, 5 mM EDTA), centrifuged as described above and resuspended in 1 ml reaction buffer until homogeneous. Small aliquots of the microsomal fraction were frozen in liquid nitrogen and stored at -80 °C.

1.3 Using the γ -secretase inhibitor test system

The frozen microsomal fraction of the induced H4-ind/APP-LC99 cells was thawed on ice and 10 μl aliquots were used for each cell-free reaction. The samples were diluted to 30 μl with reaction buffer and incubated at specific temperatures and pH values and for specific times.

After incubation the samples were adjusted to 2 % SDS and heated to 95 °C for 5 minutes. 1 ml IP buffer (150 mM NaCl, 10 mM Tris pH 7.4, 1 mM EDTA, 0.2 % NP40 and the protease inhibitors aprotinin (10 $\mu\text{g}/\text{ml}$), leupeptin (10 $\mu\text{g}/\text{ml}$), 5 $\mu\text{g}/\text{ml}$ pepstatin, 1 mM Pefabloc) and in each case 6 $\mu\text{g}/\text{ml}$ of the specific antibodies BI.40 and BI.42 (alternative antibodies with the same effect can be obtained from QCB, Quality Control Biochemicals, Inc., Hopkinton, USA; catalogue numbers 44-348 and 44-344) were added to the samples. After one hour at 4°C, 20 μl of prewashed Gammabind-Sepharose G beads (Pharmacia Biotech) were added and incubated overnight at 4°C. The sepharose immunocomplex was washed with IP buffer and precipitated proteins were eluted with 20 ml of Tris-Bicine (Klafki *et al.*, 1996) sample buffer. The samples were separated by Tris-Bicine polyacrylamide gel electrophoresis as described (Klafki *et al.*, 1996). The highly sensitive Western Blot method described earlier was used with the antibodies

6E10 and 4G8 (Product numbers mAb 200-10 and mAb 300-10, Senetek, Great Britain; Galli *et al.*, 1998) in order to detect the immunoprecipitated A β species (Ida *et al.*, 1996).

Chemiluminescence was detected with the Western Star substrate (Tropix) and quantified with a chemiluminescence detection system supplied by BioRad.

1.4 Obtaining the cytosol from guinea pig liver cells

A postnuclear supernatant (PNS) is obtained from the liver of a guinea pig by homogenisation and centrifugation as described (Taylor *et al.*, 1997). This supernatant is applied to a sucrose step gradient (Taylor *et al.*, 1997) and centrifuged at 100,000 g and 4°C for 1.5 h. The fraction with 500 mM sucrose is diluted with 1 x KPi buffer on 250 mM sucrose and centrifuged at 200,000 g and 4°C for 20 minutes. The supernatant is the cytosol (Jones *et al.*, 1998).

1.5 Alternative method of the γ -secretase inhibitor test system with an ATP-regenerating system

A purified microsomal fraction of these recombinant H4 cells is incubated at 37 °C with a suitable buffer system (50-150 mM KCl, 1.5-5 mM of magnesium acetate, 250 mM sucrose, 20 mM Hepes pH 6.8), an ATP regenerating system (1 mM ATP, 0.1 mM GTP pH 7.0; 8 mM phosphocreatine, 31 mM creatine phosphokinase) and cytosol, which has been worked up as described in 1.2, and then the γ -secretase activity is measured by detecting the product A β by Western Blot as described above.

Example – Using the test system

1.6 Alternative working up of a microsome fraction from H4 LC99 cells

The same cell line (H4 neuroglioma cell clone with APP-LC99 construct) as described in 1.1 was used.

The H4-ind/ APP-LC99 cells were grown until confluent on 15 cm Petri dishes at 37 °C at 5% CO₂ with DMEM medium (DMEM: “Dulbecco’s Modified Eagle Medium”, sold by BioWittacker) and 10% foetal calf serum (FCS), 1% glutamine, 1% penicillin and streptomycin in the absence of doxycycline. By the removal of doxycycline the expression of the fusion protein was induced for three days. All the steps of preparing the postnuclear supernatant were carried out on ice or at 4°C. For a preparative batch 10 times 15 cm Petri dishes were processed together. After the addition of 2 ml of ice-cold PBS per Petri dish the cells were removed from

the Petri dishes with a cell scraper. All the following steps were carried out as described in Schröter *et al.* After centrifugation at 500 g for 10 min the cells were carefully resuspended in ST buffer (250 mM sucrose, 10 mM Tris pH 7.4), centrifuged again at 1400xg for 10 min and then all the cells were resuspended in 5 ml of ST buffer. The cells were homogenised using a 5 ml Potter (Braun, Melsungen) at 500 rpm and the lysing of the cells was monitored by phase contrast microscopy. The cell lysate was first centrifuged at 2000xg for 2 min to precipitate any intact cells and large cell debris. Then the supernatant was centrifuged at 4000xg for 2 min to separate off the cell membranes and cell nuclei (fraction PN). The sediment consisting of cell nuclei and plasma membranes were washed twice with ST buffer and centrifuged as described above. The supernatants were combined and centrifuged in a new container at 100,000xg for 2 min in order to eliminate mitochondria, lysosomes and endosomes (fraction EL). Finally, in order to sediment the purified microsomes, the supernatant was centrifuged at 400,000xg. In order to separate out the lysosomes and the endosomes the EL fraction was processed further. The lysosomes were burst open by a 10 minute hypotonic lysis on ice (Bohley *et al.* 1969) and then the intact endosomes were sedimented at 100,000xg for 2 min. (Lysosomes = fraction L; endosomes = fraction E).

To characterise the separation, 30µg of total protein from each fraction were placed on a polyacrylamide gel and the distribution of various marker proteins of individual compartments in the fractions was detected by the Western blot method.

The endosomal and microsomal membranes were taken up with reaction buffer (250 mM sucrose, 50 mM KCl, 2.5 mM magnesium acetate, 20 mM HEPES, pH 6.8, 5 mM EDTA) and resuspended in 1 ml reaction buffer until homogeneous. Small aliquots of the different fractions were frozen in liquid nitrogen and stored at -80 °C.

Then the γ -secretase inhibitor test system was carried out exactly as described in 1.3.

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-

Claims:

1. Process for finding substances which are capable of specifically inhibiting γ -secretase, characterised in that
 - a) purified membranes are prepared from cells which have a γ -secretase activity and contain
5 a substrate of γ -secretase,
 - b) these membranes are mixed with reaction buffer and a test substance,
 - c) this mixture is incubated under conditions in which the substrate of the γ -secretase is cleaved in the absence of the test substance,
 - d) the quantity of any cleavage product formed is determined, and
 - 10 e) the result obtained is compared with the result obtained in the absence of the test substance.
 2. Process according to claim 1, characterised in that cells are cultivated which express an endogenous polypeptide which is a substrate of γ -secretase.
 3. Process according to claim 1, characterised in that cells are cultivated which express an
15 exogenous polypeptide which is a substrate of γ -secretase.
 4. Process according to claim 3, characterised in that the expression of the exogenous polypeptide is inducible.
 5. Process according to claim 4, characterised in that the expression of the γ -secretase substrate can be induced by the removal of tetracycline or a tetracycline derivative.
 - 20 6. Process according to one of claims 1 to 5, characterised in that the γ -secretase substrate is the amyloid precursor protein or a fragment thereof.
 7. Process according to claim 6, characterised in that the fragment of the amyloid precursor protein is the C99 fragment.
 8. Process according to one of claims 1 to 5, characterised in that the γ -secretase substrate is a
25 fusion protein of the amyloid precursor protein or a fragment thereof, particularly the C99 fragment, with a reporter protein.
 9. Process according to claim 8, characterised in that the reporter protein is green fluorescent protein or a derivative thereof, luciferase, secretory alkaline phosphatase or β -galactosidase.
 10. Process according to one of claims 1 to 9, characterised in that the cells are neuronal or glial
30 in origin.
 11. Process according to claim 10, characterised in that the cells are H4 cells.
-

12. Process according to one of claims 1 to 11, characterised in that the membranes are cell membranes, preferably intracellular membranes.
 13. Process according to claim 12, characterised in that the membranes are lysosomal or endosomal membranes.
 - 5 14. Process according to claim 12, characterised in that the membranes are microsomal membranes.
 15. Process according to claim 14, characterised in that the microsomal membranes are purified by the following steps:
 - a) lysing the cells,
 - 10 b) removing the cell nuclei,
 - c) purifying over sucrose density gradients,
 - d) re-sedimenting by ultracentrifugation,
 - e) homogenising,
 - f) re-sedimenting by ultracentrifugation, and
 - 15 g) re-homogenising.
 16. Process according to one of claims 1 to 15, characterised in that the pH value of the reaction buffer is in the range from 5-10, preferably in the range from 6.0-8.0, most preferably from 6.8 to 7.4, and contains at least one membrane stabiliser as a further component.
 17. Process according to claim 16, characterised in that the membrane stabiliser is sucrose or
20 sorbitol.
 18. Process according to claim 16 or 17, characterised in that the concentration of the membrane stabiliser is between 200 and 1000 mM, preferably between 200 and 500 mM, most preferably between 200 and 300 mM .
 19. Process according to one of claims 1 to 18, characterised in that the reaction buffer
25 additionally contains a complexing agent.
 20. Process according to claim 19, characterised in that the complexing agent is EDTA, EGTA or BAPTA or a salt thereof.
 21. Process according to claim 20, characterised in that EDTA, EGTA or BAPTA or the salt thereof is present in a concentration of 0.1 to 20 mM, preferably 5 to 10 mM.
 - 30 22. Process according to one of claims 1 to 21, characterised in that additionally an ATP-regenerating system is added to the reaction mixture.
-

23. Process according to one of claims 1 to 22, characterised in that the quantity of the cleavage product is determined by immunoprecipitation or Western Blot, preferably by a combination of immunoprecipitation and Western Blot.
 24. Process according to one of claims 1 to 23, characterised in the quantity of the cleavage product is determined by ELISA.
 25. Process according to one of claims 1 to 22, characterised in that the quantity of the cleavage product is determined by mass spectrometry.
 26. Process according to one of claims 8 to 22, characterised in that the quantity of the cleavage product is determined by determining the quantity of reporter protein fused to the cleavage product.
 27. Process according to claim 26, characterised in that the quantity of the luciferase, secretory alkaline phosphatase or β -galactosidase fused to the cleavage product is determined by measuring the enzymatic activity after the addition of the substrate.
 28. Process according to claim 26, characterised in that the quantity of the green fluorescent protein or a derivative thereof is determined by measuring the intensity of the fluorescent light.
 29. Test kit for finding substances which are capable of specifically inhibiting γ -secretase, characterised in that the test kit contains at least purified membranes from cells which have a γ -secretase activity and contain a substrate of γ -secretase, and also contains a reaction buffer.
 30. Test kit according to claim 29, characterised in that the membranes are lysosomal or endosomal, preferably microsomal membranes.
 31. Test kit according to one of claims 29 to 30, characterised in that the cells exogenously express a substrate of γ -secretase.
 32. Test kit according to one of claims 29 to 31, characterised in that the reaction buffer has a pH value in the range from 5-10, preferably in the range from 6.0-8.0, most preferably in the range from 6.8 to 7.4, and contains as additional components at least one membrane stabiliser.
 33. Test kit according to claim 32, characterised in that the membrane stabiliser is sucrose or sorbitol.
 34. Test kit according to claim 32 or 33, characterised in that the concentration of the membrane stabiliser in the reaction buffer is between 200 and 1000 mM, preferably between 200 and 500 mM, most preferably between 200 and 300 mM.
-

35. Test kit according to one of claims 29 to 34, characterised in that the reaction buffer additionally contains a complexing agent.
36. Test kit according to claim 35, characterised in that the complexing agent is EDTA, EGTA or BAPTA or a salt thereof.
37. Test kit according to claim 36, characterised in that EDTA, EGTA or BAPTA or a salt thereof is present in a concentration of 0.1 to 20 mM, preferably 5 to 10 mM.
38. Test kit according to one of claims 29 to 37, characterised in that the test kit additionally contains an ATP-regenerating system.
39. Test kit according to one of claims 29 to 38, characterised in that the quantity of the cleavage product is determined by immunoprecipitation or Western Blot, preferably by a combination of immunoprecipitation and Western Blot.
40. Use of a process according to one of claims 1 to 28 or of a test kit according to one of claims 29 to 39 for finding substances which are capable of specifically inhibiting γ -secretase.
41. Substance which can be found by a process according to one of claims 1 to 28 or a test kit according to one of claims 29 to 39, characterised in that it specifically inhibits the proteolytic cleaving of a γ -secretase substrate.
42. Use of a substance according to claim 41 for preparing a medicament for treating neurodegenerative disorders, particularly Alzheimer's disease.
43. Pharmaceutical formulation which contains a substance according to claim 41.

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Fig.1

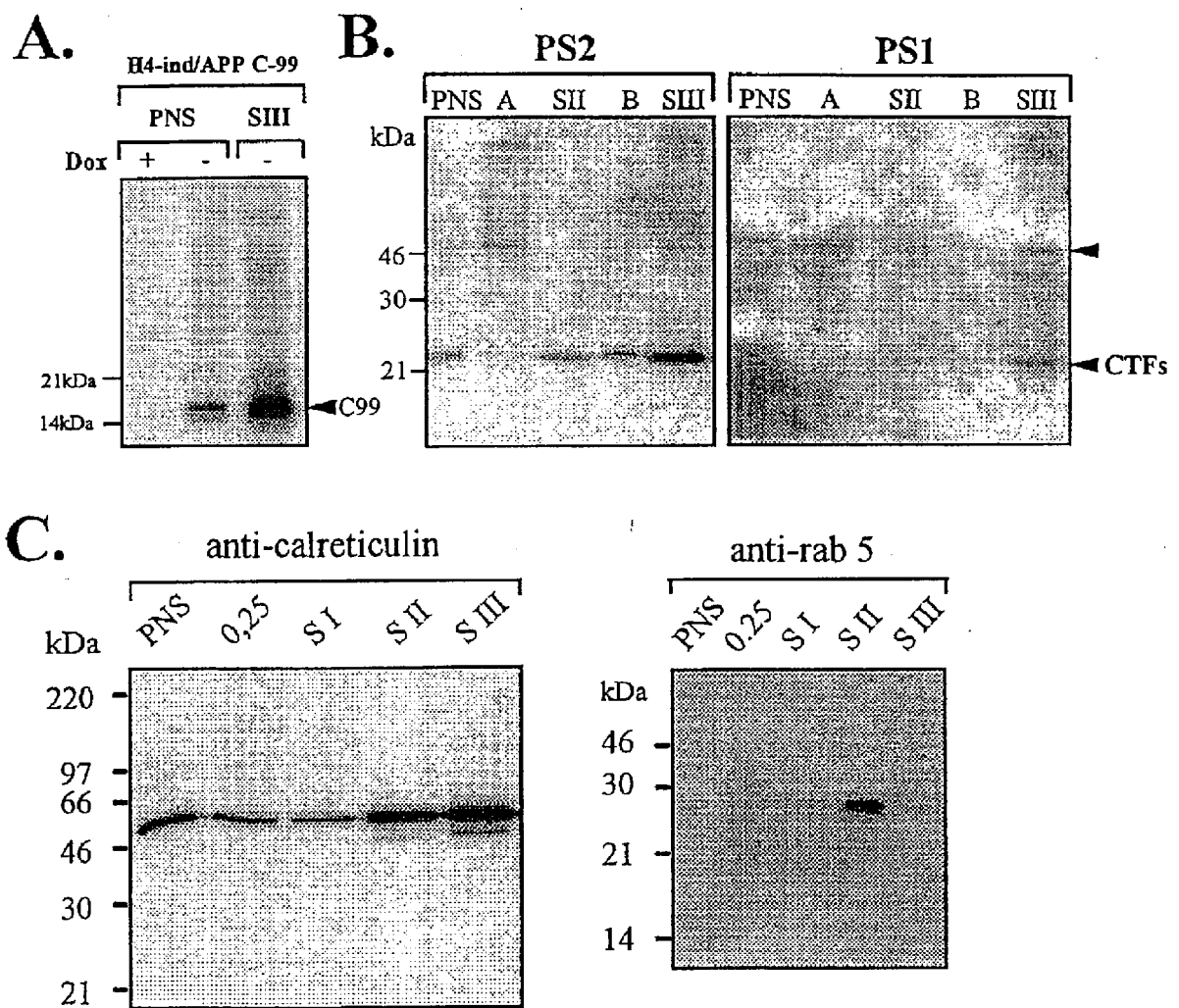
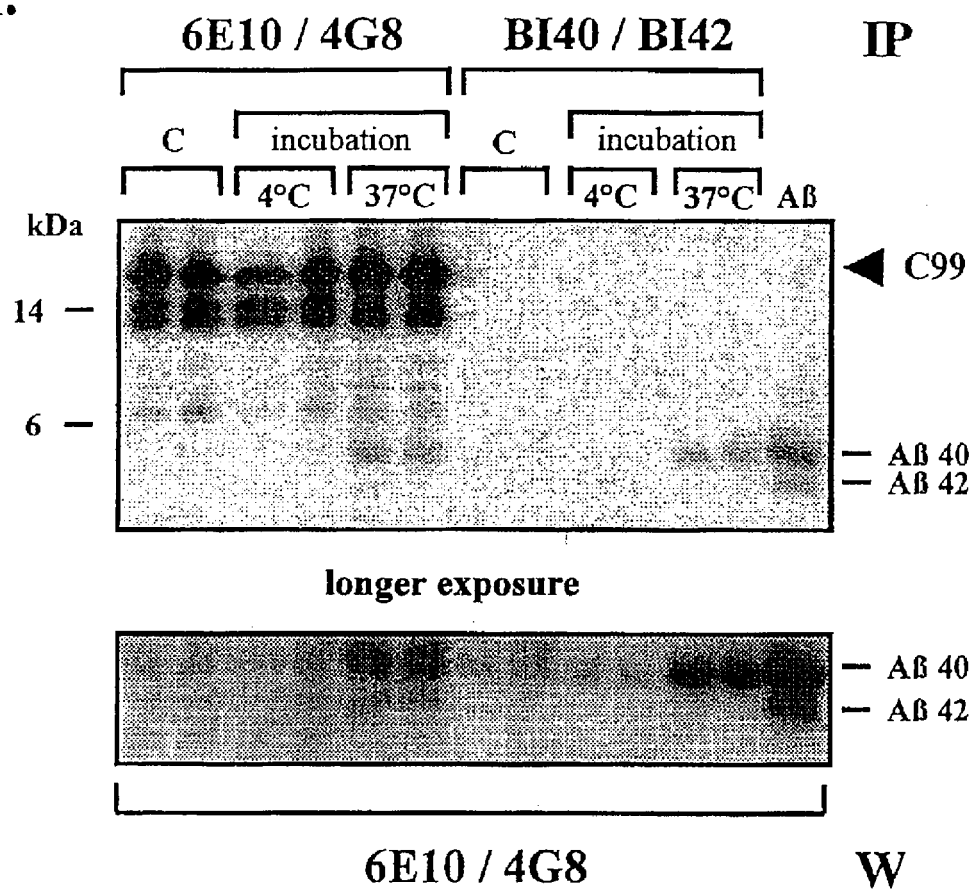


Fig.2

A.



B.

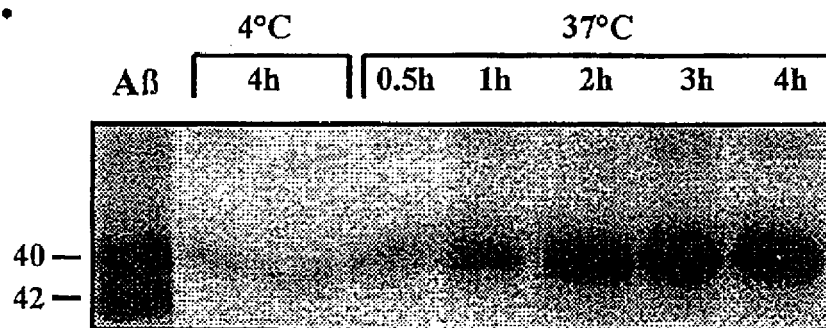


Fig.3

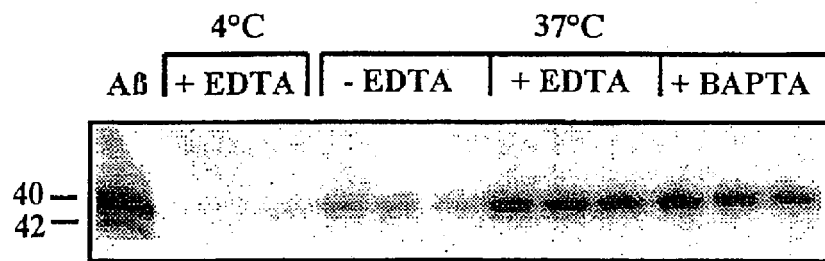


Fig.4

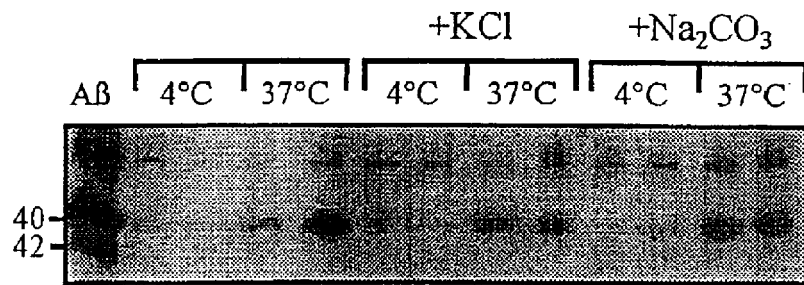


Fig.5

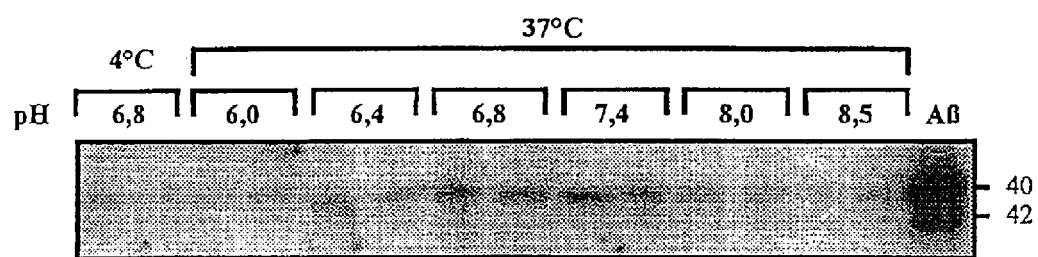
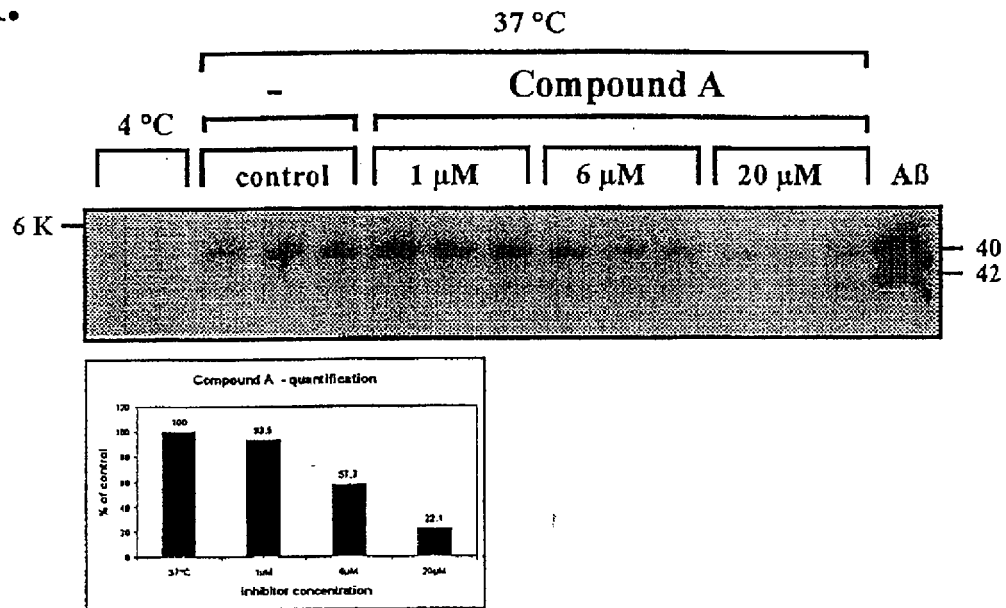


Fig.6

A.



B.

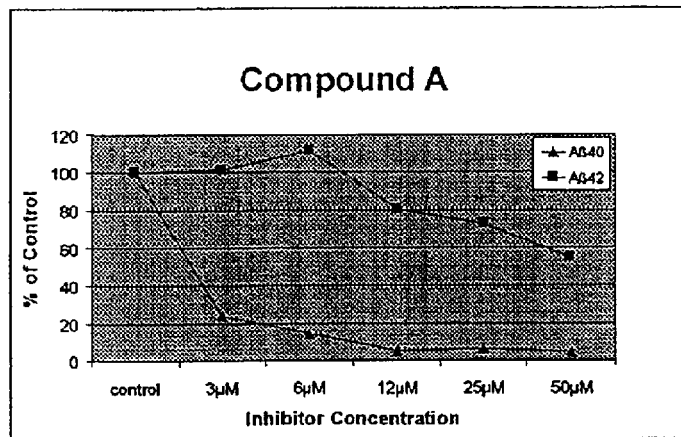


Fig. 7

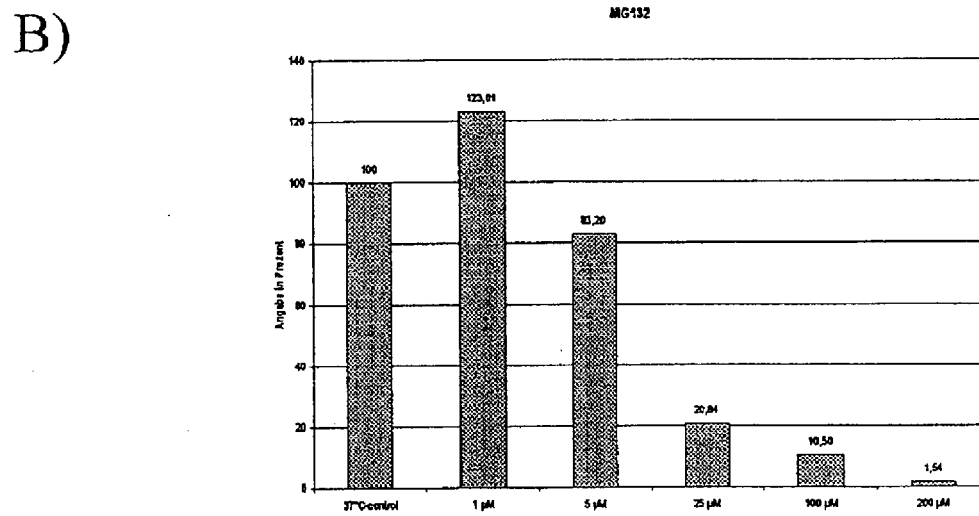
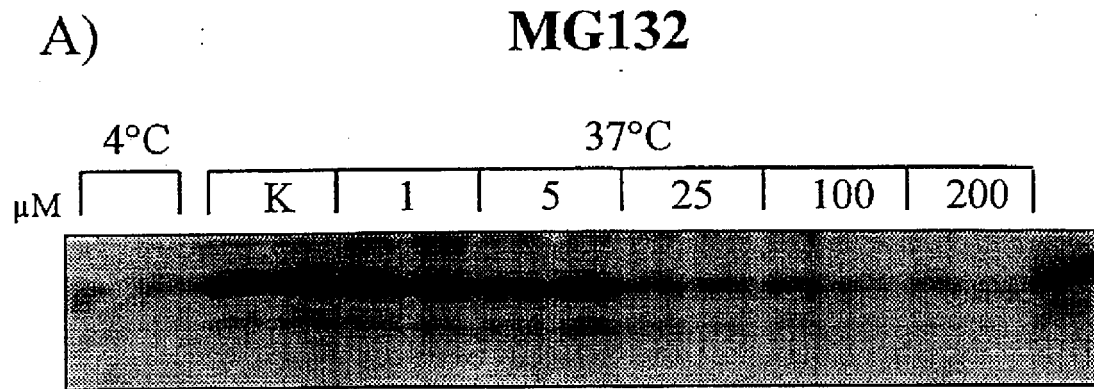
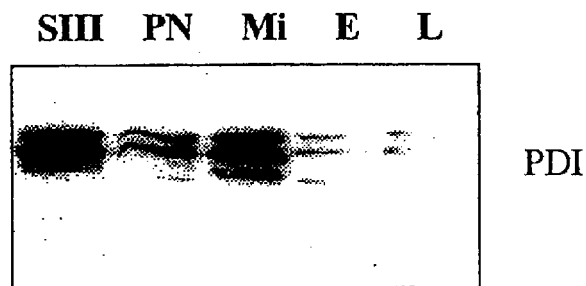
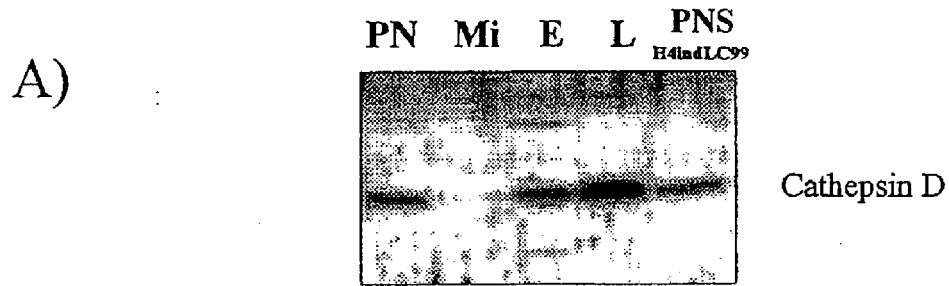


Fig. 8



B)

